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Elevated Homocysteine Levels Indicate Suboptimal Folate Status in Pediatric Sickle Cell Patients

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We investigated whether pediatric patients with sickle cell disease (SCD) (9 ± 4 years; 27 homozygous SCD [HbSS]; 19 sickle-C disease [HbSC]) have different folate status compared with age-, sex-, and race-matched normal hemoglobin (HbAA) controls (n = 20), and whether their folate status can be improved by folate supplementation. The patients were supplemented with vitamins B_6 and B_{12} during one week and with folate during the following week. Circulating folate, homocysteine, vitamin B_6 and vitamin B_{12} levels were measured at baseline (patients and controls), after one week and after two weeks (patients). The patients had similar folate, vitamin B_6, and vitamin B_{12}, but higher homocysteine levels compared with HbAA controls (12.7 ± 4.5 vs. 10.9 ± 3.5 μmol/l; $P = 0.04$). Vitamin B_6 and B_{12} supplementation did not change their homocysteine levels, but folate supplementation caused a 53% reduction (to 5.7 ± 1.6). We conclude that patients with SCD have adequate vitamin B_6 and B_{12} status, but suboptimal folate status, leading to elevated plasma homocysteine levels. They may therefore benefit from folate supplementation to reduce their high risk for endothelial damage. Am. J. Hematol. 59:192–198, 1998. © 1998 Wiley-Liss, Inc.

Key words: sickle cell disease; homocysteine; folate; endothelium

INTRODUCTION

Patients with sickle cell disease (SCD) have grossly decreased erythrocyte half-lives [1,2]. As a consequence, they have about a 3–14 times higher rate of erythropoiesis, compared with healthy controls, which may necessitate higher dietary intakes of macronutrients and especially micronutrients [3]. One of these is folate, which is involved in a number of methylation reactions including those in protein, deoxyribonucleic acid (DNA), and ribonucleic acid (RNA) synthesis. The present recommended dietary allowance (RDA) for folate amounts to 25–35 μg/day for 0–1 year infants, 50–150 μg/day for children aged 1–14 years, and 180 and 200 μg/day for women and men aged 14–51+ years, respectively [4].

Severe folate deficiency causes macrocytic anemia. There are no indications that pediatric patients with SCD need a higher folate intake to prevent the development of macrocytic anemia, at least in Jamaica [5]. Pediatric patients with SCD in the United States had folate intakes and serum and red blood cell folate concentrations comparable with controls, suggesting that routine folate supplementation is not necessary [6]. The absence of the manifest hematological signs of folate deficiency, and comparable folate intakes and status do not, however, exclude the existence of a subclinical deficiency that

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contributes to the SCD pathophysiological cascade. Currently, endothelial damage is considered to play a central role in this cascade, and suboptimal folate status may contribute to this damage by its augmentation of plasma homocysteine concentrations [7–9].

Homocysteine derives from the metabolism of the essential amino acid methionine. It is catabolized in a vitamin B_6-dependent reaction, or retroconverted to methionine by two metabolic pathways that use betaine, or both vitamin B_{12} and folate as cofactors, respectively. Hyperhomocysteinemia is a strong independent risk factor for the development of occlusive arterial and venous disease [10]. Currently, the plasma homocysteine concentration is considered to be the most sensitive functional parameter for the detection of a subclinical folate deficiency [11]. However, it is not a specific marker, since marginal vitamin B_6 and B_{12} status may also increase plasma homocysteine concentrations.

We investigated the folate status of pediatric patients with homozygous SCD (HbSS) and sickle-C disease (HbSC) by measuring both a static parameter (serum folate concentration) and a functional parameter (plasma homocysteine concentration). Folate and homocysteine concentrations were compared with those of age-, sex-, and race-matched HbAA controls. We also investigated whether the folate status of SCD patients could be improved by monitoring plasma homocysteine concentrations following oral folate supplementation. The patients initially received vitamins B_6 and B_{12} to ensure the exclusive dependence of plasma homocysteine concentrations on the folate status.

SUBJECTS AND METHODS

Study Group, Study Design, and Samples

Pediatric patients with SCD were recruited during their regular polyclinical visits at the Department of Pediatrics (St. Elisabeth Hospital, Curaçao). The number of participating patients was 27 with HbSS (aged 1–17 years; male/female = 12/15) and 19 with HbSC (aged 1–16 years; male/female = 9/10). The patients did not receive any medication or vitamins, or any blood transfusions during the preceding four months. Twenty age-, sex- and race-matched children with normal hemoglobin (HbAA) (aged 4–18 years; male/female = 10/10) were recruited from the population that visited the Public Health Laboratory for various laboratory tests as part of a routine medical checkup. We considered these patients apparently healthy and therefore suitable to serve as appropriate controls since they did not show any abnormalities in the performed standard clinical chemical tests (sodium, potassium, glucose, creatinine, cholesterol, triglycerides, and others) and hemocytometric tests.

Patients with SCD were supplemented with vitamins B_6 and B_{12} during the first week (days 0 up to, and including, day 6) and with folate during the second week (day 7 up to, and including, day 13). Vitamin B_6 (pyridoxine-hydrochloric acid, powder), vitamin B_{12} (powder), and folate (powder) were obtained from Cerrito Pharmacy (Curaçao, Netherlands Antilles). Children aged 1–6 years received total daily dosages of 125 mg of vitamin B_6 (114–125× their RDA), and one mg of vitamin B_{12} (1,000–1,430× RDA) during the first week, and two mg folate (27–40× RDA) during the second week [4]. Children aged 7–18 years received total daily dosages of 250 mg of vitamin B_6 (125–180× RDA), two mg of vitamin B_{12} (1,000–1,430× RDA), and four mg folate (20–40× RDA) [4]. The appropriate vitamin dosages were given to the parents who were instructed to dissolve the vitamins in orange juice. Vitamin B_6, folate, and half of the vitamin B_{12} dose were to be administered after dinner, whereas the other vitamin B_{12} dose was to be given after lunch.

Ethylenediaminetetraacetic acid (EDTA)-blood, blood for collection of serum, and heparin anticoagulated blood (up to five ml in total) were obtained by venipuncture in the fasting state. Samples were collected before supplementation (day 0), after vitamin B_6 and vitamin B_{12} supplementation (day 7), and after seven days of folate supplementation (day 14) from SC patients. Samples from controls were obtained by venipuncture in the fasting state for comparison with patients at baseline. Written informed consent was obtained from the parents of all patients. The study protocol was in agreement with local ethical standards and the Helsinki declaration of 1975, as revised in 1989.

Sample Processing and Analyses

Five ml EDTA-blood was used for the confirmation of the hemoglobin phenotypes with high performance liquid chromatography [12]. The remaining EDTA anticoagulated blood was immediately centrifuged (1,700*g*, 10 min, 4°C) for the analysis of homocysteine in EDTA-plasma. The plasma was stored at −70°C until transport to The Netherlands in dry ice. Analyses were done by the Clinical Chemical Laboratory (Leeuwarden), with a high performance liquid chromatographic method [13]. Heparin-blood for the analysis of vitamin B_6 was stored at −70°C until transport to The Netherlands in dry ice. Analyses were done by the Clinical Chemical Laboratory (Leeuwarden), with a high performance liquid chromatographic method [14,15]. Blood was allowed to coagulate for 10 min at room temperature for the subsequent preparation of serum by centrifugation at 1700*g* and 4°C. Serum for the analyses of vitamin B_{12} and folate was stored at −70°C until analyses in the Public Health Laboratory (Curaçao). Both vitamin B_{12} and folate were determined by competitive protein binding assays, using an immunochemistry analyzer (IMX, Abott Laboratories, IL).

TABLE I. Circulating Concentrations of Homocysteine, Vitamin B6, Vitamin B12, and Folate for Patients With HbSS and HbSC, and for HbAA Controls*

	Baseline			Sickle cell disease (n = 46)		
	HbAA (n = 20)	HbSS (n = 27)	HbSC (n = 19)	Baseline	Day 7	Day 14
Age (years)	9 ± 4 (4–18)	8 ± 4 (1–17)	9 ± 4 (1–16)	9 ± 4 (1–17)		
Male/female	10/10	12/15	9/10	21/25		
Homocysteine (μmol/l)	10.9 ± 3.5 (6.6–21.1)	12.5 ± 5.0 (8.0–33.4)	13.1 ± 3.7 (6.3–20.7)	12.6 ± 4.5 (6.3–33.4)[b]	12.5 ± 4.0 (7.9–26.8)	5.7 ± 1.6 (3.2–9.4)[e,g]
Vitamin B_6 (nmol/l)	113 ± 43 (62–231)	119 ± 38 (44–202)	95 ± 39 (23–205)[c]	110 ± 40 (23–205)	725 ± 397 (81–1860)[e]	179 ± 93 (84–610)[e,g]
Vitamin B_{12} (pmol/l)	374 ± 164 (117–667)[a]	552 ± 416 (166–2314)	482 ± 215 (280–859)	524 ± 348 (166–2314)[a,d]	1225 ± 617 (127–2989)[e]	769 ± 553 (151–3679)[f,g]
Folate (nmol/l)	18 ± 6 (9–29)	18 ± 8 (7–36)	16 ± 6 (10–30)	17 ± 7 (7–36)	15 ± 8 (5–35)[e]	61 ± 61 (15–378)[e,g]

*HbSS, homozygous sickle cell disease; HbSC, sickle-C disease; HbAA, normal hemoglobin; EDTA, ethylenediaminetetraacetic acid. Data indicate mean ± SD (range). Data of patients with sickle cell disease are pooled results of patients with HbSS and HbSC. EDTA-plasma homocysteine, heparin whole blood vitamin B_6, serum vitamin B_{12}, and serum folate were measured in the fasting state at baseline for patients with sickle cell disease and subjects with HbAA, after 7 days supplementation of the sickle cell patients with vitamins B_6 and B_{12} (indicated day 7), and after their subsequent supplementation with folate (indicated day 14). The daily vitamin dosages for patients aged 1–6 and 6–18 years were: 125 and 250 mg vitamin B_6, one and two mg vitamin B_{12}, and two and four mg folate, respectively.
[a]age dependent as established by Pearson r correlation at $P < 0.05$.
Between-group differences:
[b]$P = 0.04$ for comparison with HbAA by Mann-Whitney U test.
[c]$P = 0.02$ for comparison with HbSS by Mann-Whitney U test.
[d]$P = 0.05$ for comparison with HbAA by analysis of covariance with age as covariate.
Longitudinal differences by paired Student's *t*-test for comparisons with basline:
[e]$P < 0.0001$.
[f]$P < 0.01$.
Longitudinal differences by paired Student's *t*-test for comparisons with day 7:
[g]$P < 0.0001$.

Standard hematological and clinical chemical analyses were performed in the Public Health Laboratory with a JT Coulter Counter (Coulter International Division, Hialeah, FL) and a Beckman Synchrom CX7 chemical analyzer (Beckman Instruments, Fullerton, CA), respectively.

Data Evaluation and Statistics

We investigated age-dependency at baseline with the Pearson r-correlation, and between-group differences at baseline with the Mann-Whitney U test or with analysis of covariance (ANCOVA) (in the case of age-dependency) [16]. Correlations between plasma homocysteine and serum folate, and between the absolute decrease of homocysteine and folate were analyzed with the Spearman rank correlation test. Longitudinal changes were investigated with the paired Student's *t*-test, with Bonferroni adjustment for type-1 errors. $P < 0.05$ was considered significant.

A longitudinal change of an individual's fasting plasma homocysteine concentration was considered significant ($P < 0.05$) if the proportional difference (in %) amounted to more than 2.8 times the combined analytical and intraindividual biological coefficient of variation ($2.8*CV_{anal,biol}$) [17]. This CV has been estimated at 8.25% [18].

RESULTS

Comparison of Patients With SCD With HbAA Controls at Baseline

The baseline concentrations of homocysteine, vitamin B_6, vitamin B_{12}, and folate of subjects HbAA, HbSS, and HbSC, together with pooled data of the 46 patients with SCD are shown in Table I. Plasma vitamin B_{12} of subjects with HbAA ($r = -0.481$; $P = 0.032$) and SCD ($r = -0.315$; $P = 0.035$) proved age-dependent. Between-group comparisons for these parameters were therefore tested by ANCOVA, with age as covariate. There were no differences between the parameters mentioned above for patients with HbSS and HbSC at baseline, with the exception of vitamin B_6. We subsequently tested the pooled data of the HbSS and HbSC patients with those of the HbAA controls. Patients with SCD had higher homocysteine and vitamin B_{12} levels compared wtih HbAA controls.

Plasma homocysteine was inversely related to serum folate at baseline for patients with HbSS ($r = -0.496$; $P = 0.009$), HbSC ($r = -0.519$; $P = 0.027$), and SCD ($r = -0.503$; $P < 0.0001$). Figure 1A shows the relation of plasma homocysteine with serum folate at baseline for patients with SCD (a significant relation) and their HbAA controls (not significant). No such relations were observed in the comparisons between plasma homocysteine and serum vitamin B_{12}, or between plasma homocysteine and vitamin B_6.

Effect of Vitamin Supplementation of SCD Patients

Patients with HbSS and HbSC did not have different levels of the various parameters on days 7 and 14, except for higher vitamin B_6 in patients with HbSS on day 14 ($P = 0.006$). We subsequently tested longitudinal changes for the whole group of SCD patients (Table 1). Supplementation of vitamins B_6 and B_{12} increased their respective circulating levels on day 7, but decreased the plasma folate concentrations. There were no changes in plasma homocysteine levels. After starting folate supplementation, homocysteine, vitamin B_6, and vitamin B_{12} levels decreased from day 7 to day 14, whereas folate levels increased. Vitamin B_6, vitamin B_{12}, and folate levels were higher on day 14 compared with day 0, whereas homocysteine levels were lower. The number of SCD patients that exhibited significant individual decreases of plasma homocysteine from day 0 to day 7 was five (12.2%), whereas the number that showed increases was four (9.8%). All patients showed significant plasma homocysteine decreases from day 7 to day 14.

The relation between the absolute plasma homocysteine decline from day 7 to day 14 with the serum folate concentration at baseline is shown in Figure 1B. This relation indicates the dependency of the absolute homocysteine decline following folate supplementation on the initial serum folate concentration, which proved to be significant with a correlation coefficient of 0.434 ($P = 0.005$). The correlation was found to be significant for patients with HbSS ($r = -0.457$; $P < 0.025$) but not for patients with HbSC.

DISCUSSION

We investigated whether patients with HbSS and HbSC have different folate status compared with age-, sex-, and race-matched HbAA controls, and whether their folate status can be improved by folate supplementation. We evaluated both a static marker (serum folate) and a sensitive functional marker (plasma homocysteine) of folate status. To eliminate possible confounding influences on the plasma homocysteine concentrations, patients were supplemented with vitamins B_6 and B_{12} before folate administration. Our results show that patients with HbSS and HbSC do not have different serum folate levels, but that they have higher plasma homocysteine levels compared with HbAA controls (Table 1). Their plasma homocysteine proved independent of the vitamin B_6 and B_{12} status, and supplementation of these vitamins did not change their plasma homocysteine levels either, indicating an adequate status of these vitamins. However,

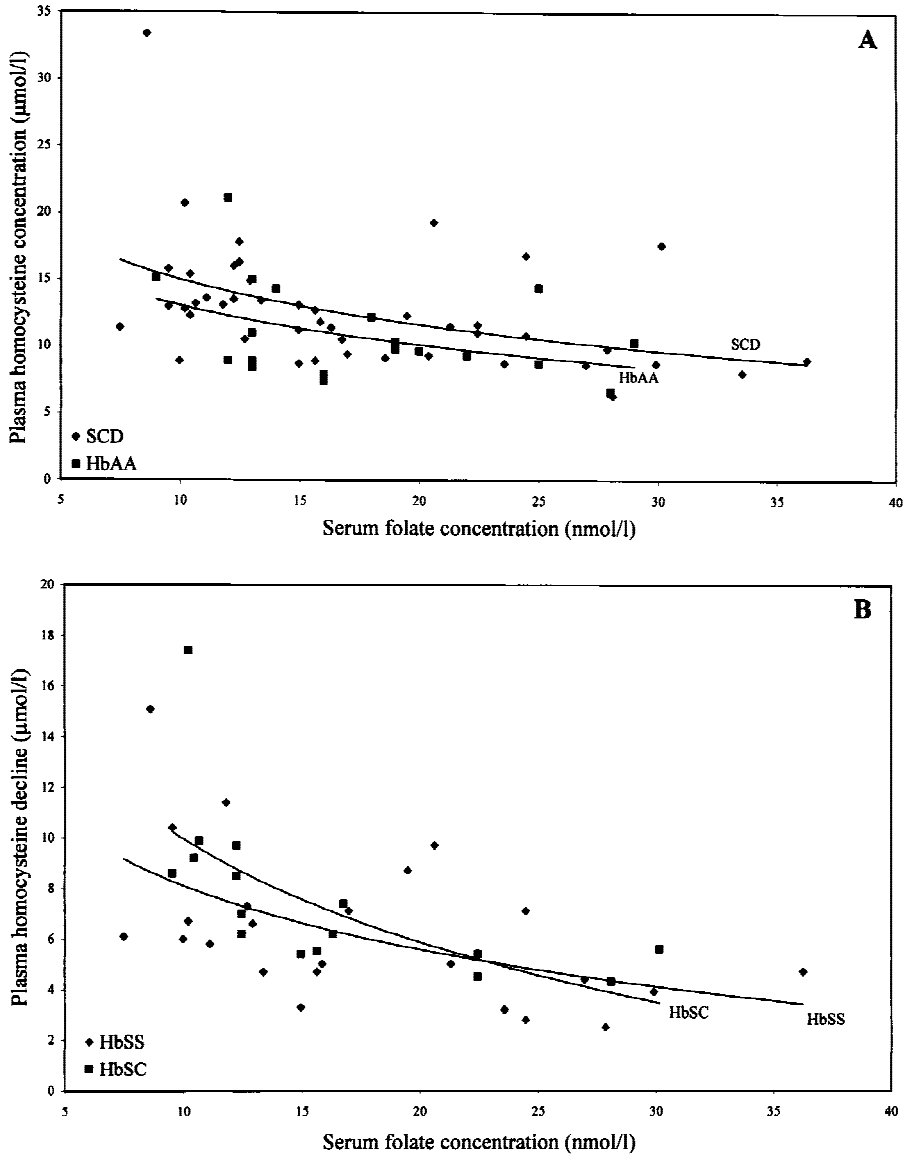

Fig. 1. Relation between plasma homocysteine and serum folate at baseline (A), and between the decline of plasma homocysteine from day 7 to day 14 and serum folate on day 7 (B). For study design see legend of Table I. A: Data derived from 45 patients with SCD (27 HbSS, 18 HbSC) and 20 HbAA controls at baseline (day 0). The relations were fitted into logarithmic curves. Plasma homocysteine was inversely related to serum folate for patients with SCD (Spearman rank correlation: r = −0.503; *P* < 0.001) and HbAA controls (not significant). B: Data derived from 41 patients with SCD (24 HbSS, 17 HbSC). The relations were fitted into logarithmic curves. Serum folate was measured on day 7. The plasma homocysteine decline indicates the decline from day 7 to day 14 (i.e., after one week of folate supplementation). The plasma homocysteine decline was inversely related to the initial serum folate concentration (Spearman rank correlation: r = −0.434; *P* = 0.005 for all 41 patients).

their plasma homocysteine concentration was inversely related to their serum folate concentration (Fig. 1A). Supplementation with folate revealed that their folate status can be improved considerably, since it caused a 53% reduction of their plasma homocysteine concentrations (Table 1); also, the magnitude of their absolute homocysteine decrease proved to be related to their initial folate concentration (Fig. 1B).

The 53% decline of the plasma homocysteine concentration (Table 1: from 12.6 to 5.7 μmol/l) is much steeper compared with the homocysteine decline (about 22%) of both apparently healthy adults in Denmark (From 8.79 to 6.88 μmol/l) and The Netherlands (from 11.7 to 9.1 μmol/l) [19,20]. The big difference in the plasma homocysteine following folate supplementation in the present study is notably caused by a much lower plasma homocysteine concentration after folate supplementation and to a lesser extent by a difference in the initial plasma homocysteine concentration. Lower homocysteine concentration following folate supplementation of blacks has been noted previously [21]. The steep homocysteine decline of the SCD patients in Curaçao proved unpredictable from their serum folate concentrations at baseline conditions (all subjects in present study: 17 ± 7 nmol/l) since these are comparable with those of apparently healthy adults in The Netherlands (14 ± 8 nmol/l) [21]. Therefore, serum folate seems to be a poor predictor of the lowest homocysteine concentrations that can be reached in a given population upon folate supplementation. Measurement of plasma homocysteine may provide a better reflection of the folate status than the measurement of plasma folate. It should be remembered, however, that plasma homocysteine is also dependent on the vitamin B_6 and B_{12} status.

The higher plasma homocysteine levels of SCD patients, and the fact that these patients do not have different serum folate levels compared with controls, suggest that they need a higher plasma folate concentration to reach plasma homocysteine levels similar to those of HbAA controls. This may be related to their higher erythrocyte turnover or to an impaired red blood cell folate metabolism (e.g., due to sickling), but other explanations, such as decreased renal function, cannot be ruled out [10,22]. Nevertheless, having the lowest possible plasma homocysteine levels may be advisable since the risk of vascular disease increases continuously with increasing plasma homocysteine levels [10]. Homocysteine is assumed to affect the vascular endothelium, as well as platelets and coagulation proteins [10,23]. Current concepts in the pathophysiology of SCD show a prominent role of dysfunctional endothelium in the pathophysiology of sickle cell vasoocclusion [7,8]. Although no effect of folate supplementation on the frequency of painful crises has been reported, the contribution of elevated homocysteine levels to endothelial damage may be an additional and easily avoidable risk factor, next to the apparently unavoidable devastating effects of the erythrocyte sickling cycle [5,9,24]. Our results show that similar folate levels compared with healthy controls do not exclude the existence of a suboptimal folate status in children with SCD. Therefore, folate supplementation of patients with SCD seems indicated, although the (long term) benefits of such a strategy remain as yet to be established in a randomized trial. We are currently determining the minimal daily folate dose required to achieve a similar homocysteine reduction as described here.

CONCLUSION

We report that patients with SCD have serum folate, whole blood vitamin B_6, and serum vitamin B_{12} concentrations that are comparable with age-, sex- and race-matched HbAA controls. Supplementation of SCD patients with vitamins B_6 and B_{12} did not cause plasma homocysteine changes, which suggests that the status of these vitamins is adequate. Patients with SCD, however, do have higher baseline plasma homocysteine concentrations, and folate supplementation caused a 53% reduction of their plasma homocysteine levels. This suggests that they have less optimal folate status compared with healthy counterparts, and that they may benefit from folate supplementation to reduce their high risk for endothelial damage.

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